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Inhibition of the α-ketoglutarate dehydrogenase complex alters mitochondrial function and cellular calcium regulation

Hsueh-Meei Huang*, Hui Zhang, Hui Xu, Gary E. Gibson

Dementia Research Service, Weill Medical College of Cornell University, Burke Medical Res. Institute, 785 Mamaroneck Avenue, White Plains, NY, 10605, USA

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Abstract

Mitochondrial dysfunction occurs in many neurodegenerative diseases. The α-ketoglutarate dehydrogenase complex (KGDHC) catalyzes a key and arguably rate-limiting step of the tricarboxylic acid cycle (TCA). A reduction in the activity of the KGDHC occurs in brains and cells of patients with many of these disorders and may underlie the abnormal mitochondrial function. Abnormalities in calcium homeostasis also occur in fibroblasts from Alzheimer's disease (AD) patients and in cells bearing mutations that lead to AD. Thus, the present studies test whether the reduction of KGDHC activity can lead to the alterations in mitochondrial function and calcium homeostasis. α-Keto-β-methyl-*n*-valeric acid (KMV) inhibits KGDHC activity in living N2a cells in a dose- and time-dependent manner. Surprisingly, concentration of KMV that inhibit in situ KGDHC by 80% does not alter the mitochondrial membrane potential (MMP). However, similar concentrations of KMV induce the release of cytochrome *c* from mitochondria into the cytosol, reduce basal $[Ca^{2+}]_i$ by 23% ($P<0.005$), and diminish the bradykinin (BK)-induced calcium release from the endoplasmic reticulum (ER) by 46% ($P<0.005$). This result suggests that diminished KGDHC activities do not lead to the Ca^{2+} abnormalities in fibroblasts from AD patients or cells bearing PS-1 mutations. The increased release of cytochrome *c* with diminished KGDHC activities will be expected to activate other pathways including cell death cascades. Reductions in this key mitochondrial enzyme will likely make the cells more vulnerable to metabolic insults that promote cell death.

Keywords: Mitochondrial membrane potential; α-keto-β-methyl-*n*-valeric acid; Neuroblastoma; Cytochrome *c*; Endoplasmic reticulum; Calcium

1. Introduction

Considerable evidence implicates mitochondrial dysfunction in several neurodegenerative diseases [1,2]. The α-ketoglutarate dehydrogenase complex (KGDHC) is a mitochondrial enzyme complex that oxidatively decarboxylates α-ketoglutarate to succinyl-CoA in the tricarboxylic acid cycle (TCA). Diminished KGDHC activities (39–83%) occur in Alzheimer's disease (AD) in both histopathologically affected and histopathologically unaffected areas of brain. This finding has been confirmed in four independent laboratories [3–6], and there are no contravening reports. KGDHC activity is reduced in fibroblasts from AD patients [7]. KGDHC activity is also reduced in other neurodegenerative diseases, including Parkinson's disease [8–11], Wernicke–Korsakoff syndrome [12], spinocerebellar disorders [13] and progressive supranuclear palsy [14,15]. Thus, diminished KGDHC activity may underlie the mitochondrial abnormality in numerous disorders.

Another irregularity that occurs in cells from AD patients is abnormal calcium homeostasis [16,17]. Bombesin-induced Ca^{2+} release from the endoplasmic reticulum (ER), which is inositol trisphosphate-mediated, is greatly enhanced in AD fibroblasts [16,18,19]. Furthermore, bradykinin (BK), another activator of phospholipase C, elicits similar enhancement of Ca^{2+} signaling in AD fibroblasts [16,18,19]. Whether this change is related to the reduction in KGDHC activities that is discussed in the first paragraph is unknown. Thus, the goal of the current study was to

Abbreviations: BSS, Balanced salt solution; BK, bradykinin; DMEM, Dulbecco's modified Eagle's medium; EPR, exhaustive photon reassignment; ER, endoplasmic reticulum; fura-2AM, fura-2-acetoxymethyl ester; KGDHC, α-ketoglutarate dehydrogenase complex; KMV, α-keto-β-methyl-*n*-valeric acid; MMP, mitochondrial membrane potential; MLP, mitochondria-like particles; PBS, phosphate-buffered saline; PSF, point spread function; TCA, tricarboxylic acid cycle; TMRM, tetramethylrhodamine methyl ester; $[Ca^{2+}]_i$, cytosolic free calcium concentration

* Corresponding author. Tel.: +1-914-597-2294; fax: +1-914-597-2757.

E-mail address: hhuang@burke.org (H.-M. Huang).

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determine if reduced KGDHC activities could lead to the alterations in calcium homeostasis.

The mitochondrial membrane potential (MMP) provides a sensitive measure of mitochondrial function. The MMP is synonymous with the electrical membrane potential that modulates Ca^{2+} regulation [20–22], and production of reactive oxygen species [23,24]. The proton electrochemical gradient potential, which controls ATP synthesis and respiration, is the sum of the MMP and the proton gradient. Since the pH component is generally small, MMP reflects the functional status of mitochondria [25,26]. Accurate assessment of the MMP within living cells under physiological conditions is essential to our understanding of the role of mitochondria in altered cell function. The experiments in this paper use a unique method that complements investigations that estimate MMP of individual mitochondrion [27–29] and those that assess MMP by examining global fluorescence from cells [30–32]. The method utilizes tetramethylrhodamine methyl ester (TMRM) at concentrations and exposure times that minimize phototoxicity and assure that changes in intensity reflect a change in the MMP [33].

In addition to their role in maintaining cellular ATP and regulating cellular calcium, mitochondria regulate apoptotic cascades in the cells by release of cytochrome *c*. The relationship between the release of cytochrome *c* and MMP is controversial. Cytochrome *c* is located on the inner membrane of mitochondria and links complex III with cytochrome *c* oxidase. Some reports suggest that the release of cytochrome *c* from mitochondria depends on the reduction of MMP [34–38] and/or induction of mitochondrial permeability transition (MPT) [39,40]. On the other hand, others suggest that the release of cytochrome *c* occurs without depolarization of MMP [30,41–44] or the MPT [45].

ER and mitochondria are intimately connected. Mitochondrial Ca^{2+} not only regulates mitochondrial metabolism and transient MMP but also regulates cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and the ER Ca^{2+} store [46–48]. Mitochondria regulate $[Ca^{2+}]_i$ through the local regulation of IP_3-sensitive channels in the ER [48,49]. These microdomains allow rapid uptake of Ca^{2+} by mitochondria prior to release in cytosol. Mitochondria take up the Ca^{2+} when $[Ca^{2+}]_i$ is above the set point and release it back into the cytosol when the $[Ca^{2+}]_i$ is below this set value [46–48]. Mitochondria also regulate $[Ca^{2+}]_i$ and ER Ca^{2+} stores by altering the level of ATP, which may affect the activity of ion pumps responsible for removing Ca^{2+} from the cytosol either to the extracellular space or into the ER [50]. Therefore, altered mitochondrial bioenergetics will change $[Ca^{2+}]_i$ and ER Ca^{2+} stores. Increases in Ca^{2+} concentrations in response to stimulus are reduced in both cytosol and mitochondria under conditions that lead to apoptosis [51,52].

The goal of the current studies was to test whether reduced KGDHC activities could lead to changes in mitochondrial function (including measures of bioenergetics and release of signaling molecules) and to alterations in calcium that occur in fibroblasts from AD patients. KGDHC was inhibited with α-keto-β-methyl-*n*-valeric acid (KMV), a structural analogue of α-ketoglutarate, that is a relatively specific competitive inhibitor of KGDHC [54]. For example, 20-mM KMV inhibits isolated rat brain KGDHC [54] or KGDHC in isolated mitochondria [54] by 99%. On the other hand, KMV only slightly inhibits (12% or less) [53] or does not inhibit [55] the pyruvate dehydrogenase complex, a very similar enzyme. Thus, KMV, which effectively and relatively specifically inhibits KGDHC activity, was utilized to test the relation of KGDHC inhibition and MMP to the release of cytochrome *c* and calcium homeostasis in N2a neuroblastoma cells.

2. Materials and methods

The supplies were obtained from the indicated companies: all of the enzymes, substrates and KMV (Sigma Chemical Co., St. Louis, MO), fura-2-acetoxymethyl ester (fura-2AM) and TMRM (Molecular Probes, Eugene, OR), cell culture reagents (GIBCO, Grand Island, NY), mouse monoclonal anti-cytochrome *c* antibody (BD PharMingen, San Diego, CA), horseradish peroxidase (HRP)-conjugated anti-mouse antibodies (Jackson Immuno-Research Lab., West Grove, PA), and anti-mouse tubulin mAb (Promega, Madison, WI).

2.1. Cultured cells

N2a neuroblastoma cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained at 37 °C in a humidified incubator with 5% CO_2 and 95% air in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were subcultured twice a week.

2.2. In situ KGDHC activity

The in situ activity of KGDHC was measured in intact living cells by the formation of a dark blue formazan product, which was produced from an electron transfer from NADH to nitroblue tetrazolium (NBT) [56]. Since other cellular dehydrogenases (e.g. pyruvate dehydrogenase complex or glutamate dehydrogenase) also utilize NAD^+, the specificity of the in situ staining for KGDHC was determined by subtracting the background in the absence of substrate α-KG and Co-A. N2a cells were seeded at a density of 10^5 cell/well in 24-well plates pre-coated with poly-D-lysine (5 μg/ml) 2 days before the experiments. On the day of experiment, the medium was aspirated, and the cells were washed with balanced salt solution (BSS in mM: NaCl 140, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, glucose 5, HEPES 10, pH 7.4 at 371 °C). The cells were then incubated with or without KMV in BSS for the indicated times. At the end of treatment the medium was changed to assay media as described previously [56] with or

without KMV. Since KMV is a reversible inhibitor for KGDHC, it was also included during the assay. After incubation, the treatment medium was aspirated, and the cells were washed with Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (HBSS). The dark blue formazan product was solubilized with SDS (10%) in 0.01 N HCl overnight in the CO_2 incubator (37 °C). The absorbance was read at 570 nm with Spectra Max 250 model plate reader (Molecular Devices; Sunnyvale, CA).

2.3. Mitochondrial membrane potential

Accurate assessment of the MMP within living cells under physiological conditions (37 °C) is essential to understand the role of mitochondria in altered cell function following inhibition of mitochondrial enzymes. Our recently published method provides a quantitative estimate of the MMP of populations of mitochondria-like particles (MLP) in a single cell under physiological condition. The method allows us to study the heterogeneity of the MMP of mitochondria (MLP) within individual cells and between cells. Changes in the MMP of MLP as small as 5% in either direction are detectable. This method combines conventional fluorescence microscopy and 3-D deconvolution by exhaustive photon reassignment (EPR) (Scanalytics, Fairfax, VA). We have optimized the dye concentrations and exposure times for the fluorescent signal of TMRM [33]. The ability to determine small changes in the MMP in depolarization or hyperpolarization suggests that self-quenching and matrix aggregation of the dye were not a problem. The stability of the MMP in controls during the time of incubations indicates phototoxicity is not a problem.

A brief description of the method follows. N2a cells were seeded on poly-D-lysine pre-coated Delta T 3.5-cm dishes at 4×10^4/dish (Bioptechs; Butler, PA) overnight. On the day of experiment, cells were loaded with TMRM (50 nM) in a BSS for 40 min at 37 °C. Stacks of fluorescence images were obtained with excitation at 540 nm and emission at 590 nm with a Delta Scan System from Photon Technology International (PTI, Lawrenceville, NJ; xenon light source, 75 W) equipped with a 12-bit digital cooled CCD Quantix camera (Photometrics, Tucson, AZ). Stacks of fluorescence images with 700-ms light exposure each, 0.25 μm per step, were taken at different time intervals. Point spread functions (PSF) to correct for light from other planes by EPR (Scanalytics) were acquired under the same conditions as the samples.

The images were analyzed by a combination of Metamorph and Microsoft Excel. This method utilizes the ratio of the TMRM signal from the mitochondria to that of the cytosol. The cytosolic values are taken to be equal to that of the nucleus. The mitochondrial values are taken to be the values that exceed a threshold of the nuclear signal plus 3 standard deviations. An MLP was defined as a group of two or more adjacent pixels that exceeded the threshold. A numerical estimate of the MMP was obtained by inserting the ratio of mitochondrial fluorescence to that of the cytosol in the Nernst equation. The average MMP of all MLP for each cell (mean MMP) was calculated. An alternative way of analyzing these data is to regard each pixel that exceeds the threshold as an independent signal, calculate its potential and sum of the potentials of all pixels for each cell.

2.4. Accumulation of cytochrome c in the cytosol

N2a cells were seeded at 6×10^6 cells per 10-cm dish 1 day before the experiment. On the day of experiment, the medium was aspirated, and the cells were washed once with phosphate-buffered saline (PBS). Cells were treated with or without KMV (20 mM) in BSS for 80 min at 37 °C. After treatment, the mitochondrial and cytosolic fractions were isolated by modifications of published methods [57]. Cells were washed with ice-cold PBS and then incubated in 0.3 ml of lysis buffer (in mM: HEPES–KOH, 20, pH 7.5; $MgCl_2$, 1.5; EDTA, 2; EGTA, 5; dithiothreitol, 0.1; phenylmethylsulfonylfluoride, 0.1; sucrose 250) on ice for 30 min before scraping. Cell lysates were homogenized in Eppendorf tubes with 10 strokes of a motorized homogenizer, and then centrifuged at $500 \times g$ for 10 min to remove unbroken cells and nuclei. Supernatants were centrifuged at $10{,}000 \times g$ for 30 min. The pellets (i.e. mitochondrial fraction) were resuspended in lysis buffer. The supernatants of the $10{,}000 \times g$ spin were further centrifuged at $100{,}000 \times g$ (BECKMAN TLX ultracentrifuge) for 60 min to yield cytosolic fractions.

Twenty micrograms (mitochondria) or 40 μg (cytosol) of protein was loaded to each lane of a 15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis. Resolved proteins were electroblotted onto PVDF transfer membranes (NEN; Boston, MA). The blots were incubated with Tris-buffered saline-Tween (TBST; 0.1 M Tris buffer, pH 7.4, 0.9% NaCl, 0.1% Tween 20) and 5% skim milk containing anti-cytochrome *c* (1:500; BD PharMingen Tech) or anti-tubulin (1:2000; Promega) monoclonal antibody overnight. Blots were then incubated with horseradish peroxidase conjugated affinity goat anti-mouse IgG (1:5000; Jackson) for 1 h. The bound immunoproteins were detected by enhanced chemiluminescent assay (ECL, Amersham Pharmarcia Biotech; Piscataway, NJ). Tubulin protein was also determined in each blot to demonstrate that equal protein was loaded on each lane. The immunoactivites of the bands were semi-quantified by densitometeric scanner and analyzed by Quantity-One software (BioRad; Hercules, CA).

2.5. Measurement of $[Ca^{2+}]_i$

Fluorescence intracellular calcium images were monitored according to our published method [18]. N2a cells were loaded with 2 μM Fura-2 AM in BSS for 1 h at 37 °C in a 5% CO_2 incubator. Cells were rinsed twice and $[Ca^{2+}]_i$ was monitored on the stage of an inverted Olympus IX70 microscope at 37 °C with a Delta Scan System from PTI. Excitation wavelengths were altered between 350 and 378

nm (band pass 3 nm) and emission was monitored at 510 nm with a Hamamatsu C2400SIT camera at 5-s intervals. Basal $[Ca^{2+}]_i$ was measured for 1 min before the addition of BK (10 nM). Then the BK-stimulated increase in $[Ca^{2+}]_i$ was measured for 4 min. Each value was the average of 32 images taken within 5 s. Standard images of Fura 2 solutions with minimum and maximum $[Ca^{2+}]_i$ were taken at the end of each day's experiment to calculate the intracellular calcium concentrations.

2.6. Statistical analysis

All data were expressed as mean ± S.E. For single variable comparisons, Student's *t* test was used. For multiple variable comparisons, data were analyzed by one-way analysis of variance (ANOVA) followed by a Student–Newman–Keul's test.

3. Results

3.1. KMV inhibited in situ KGDHC activity

To determine the degree of inhibition by KMV, in situ KGDHC activity in living N2a cells was assessed with a histochemical assay [56]. N2a cells were treated with different concentrations of KMV (0, 1, 10 and 20 mM) for the indicated times (40, 60, 80, 120 and 240 min) (Fig. 1). KMV

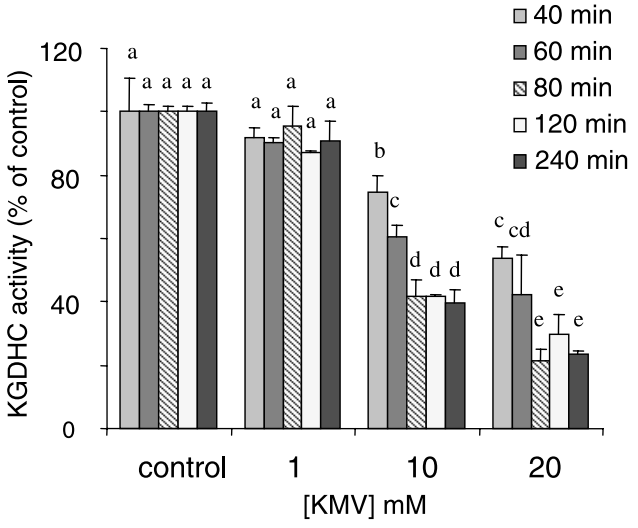

Fig. 1. KMV inhibited in situ KGDHC activity. N2a cells (10^5 cell/well) were seeded into each well of a 24-well plate 1 day prior to the experiment. On the day of experiment, the cells were washed twice with BSS, and then pretreated with different concentrations of KMV (0, 1, 10 and 20 mM) for the indicated times (0, 20, 40, 80 and 200 min) prior to the histochemical KGDHC assay which was done in the presence of KMV for 40 min (see Section 2). Thus, total treatment times with KMV were 40, 60, 80, 120 and 240 min. Each value represents the mean ± S.E. of two separate experiments done in three to four wells. Values with different letters vary significantly from each other ($P<0.05$) by ANOVA followed by Student–Newman–Keul's test.

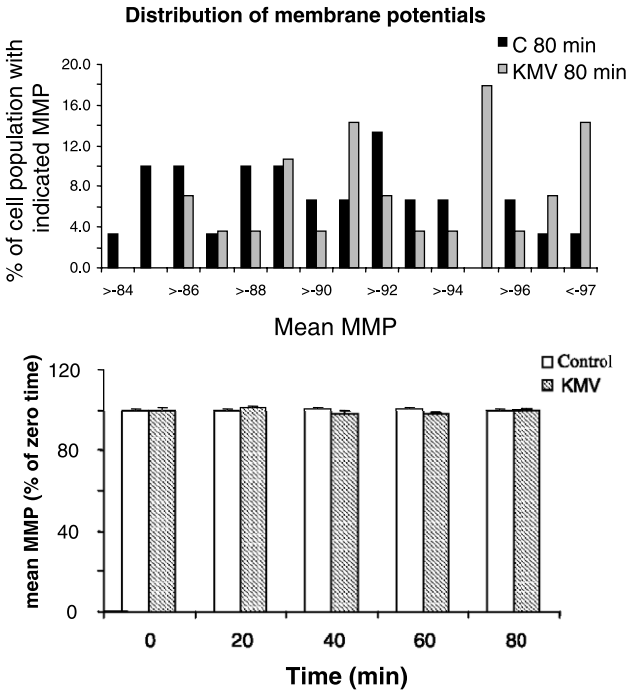

Fig. 2. Effects of KMV on distribution and temporal response of mitochondrial membrane potentials (MMP). N2a cells were seeded on delta T dishes 1 day before the experiment. On the day of experiment, N2a cells were loaded with TMRM (50 nM) in BSS for 60 min at 37 °C, and then the dishes were transferred to the stage of microscope and maintained at 37 °C. The cells were treated with KMV (20 mM) or control buffer and images were acquired at 0, 20, 40, 60 and 80 min. Top: Distribution of mean MMP in cells treated with ($n=29$) or without ($n=30$) KMV for 80 min. Values on the vertical axis are the percent of the cell population with the indicated mean MMP. Bottom: Mean cellular MMP ± S.E. after KMV treatment for different times. Values are presented as a percent of each cell's value at zero time.

inhibited KGDHC activities in a dose- and time-dependent manner. Even a 4-h incubation with 1 mM KMV did not reduce the in situ KGDHC activity. KMV at 10 and 20 mM for 40 min inhibited KGDHC activities by 25 ± 5% and 46 ± 3%, respectively. KMV at 10 and 20 mM for 80 min decreased KGDHC activity by 58 ± 5% and 80 ± 3.6%, respectively. Longer incubation times (120 and 240 min) at these higher KMV concentrations did not inhibit the enzyme activity further. Thus, experiments on the release of cytochrome *c* and calcium homeostasis were done with 20 mM KMV for 80 min.

3.2. Effect of KMV on MMP in N2a cells

To determine whether diminished KGDHC activity altered the bioenergetics of mitochondria, MMP were measured. This approach, which is described in Section 2, allows us to assess heterogeneity of MMP for individual MLP between cells. KMV did not alter several measures of the MMP of cells. The mean MMP showed considerable heterogeneity between cells under control conditions and following treatment with KMV (Fig. 2, top). The mean

MMP range of the cells varied from −84 to −97 mV. KMV did not alter distribution of the MMP of cells. The average MMP of all MLP for each cell (mean MMP) was not altered by KMV at any incubation time. If each cell's mean MMP was expressed as a percent of its value at zero time, KMV had no effect (Fig. 2, bottom).

Alternative analysis of the fluorescent signals from TMRM in the same cells provides greater power to distinguish small metabolic perturbations [33]. A method for detecting small differences is to calculate the MMP for each pixel that exceeds the threshold and sum the values of MMP for each cell. This approach did not reveal any differences between the control and the KMV group at 20, 40, 60 and 80 min (analysis not shown).

3.3. KMV induced accumulation of cytochrome c in the cytosol

To test whether partial reduction of KGDHC induces cytochrome *c* release from mitochondria in N2a cells, cells were treated without or with KMV (20 mM) for 80 min. After treatment, the protein levels of cytochrome *c* in the cytosolic and mitochondrial fractions were determined (Fig. 3). Tubulin protein levels in the same blot were used as a controls to show equivalent loading of protein on lanes that were compared for cytochrome *c*. Inhibition of KGDHC by KMV for 80 min increased accumulation of cytochrome *c* in the cytosol by 161% (Fig. 3).

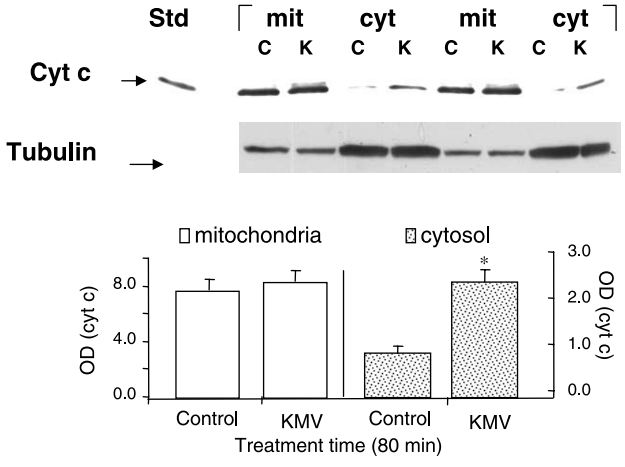

Fig. 3. KMV induced the accumulation of cytochrome *c* in the cytosol. The N2a cells were treated with KMV (20 mM) or control buffer for 80 min. The mitochondrial and cytosolic fractions were obtained according to the procedures described in Section 2. Cytochrome *c* protein levels in mitochondrial (20 μg) and cytosolic (40 μg) fractions were determined by immunoblotting. Tubulin protein levels in the same blot were determined as a control to assure that the levels of protein loading level on each lane were coordinated. The bound immunoproteins were detected by ECL assay. The top panel represents a typical immunoblot of cytochrome *c* in mitochondrial (mit) and cytosolic fractions (cyt) after the cells were treated either without (controls; C) or with KMV (K) was shown. The bottom panel represents the relative intensities of the bands after they were semiquantified with a densitometeric scanner and analyzed by Quantity-One software. Values are means ± S.E. from two separate experiments and three blots from each. * Denotes KMV differs significantly from the control ($P<0.05$) by Student–Newman–Keul's test.

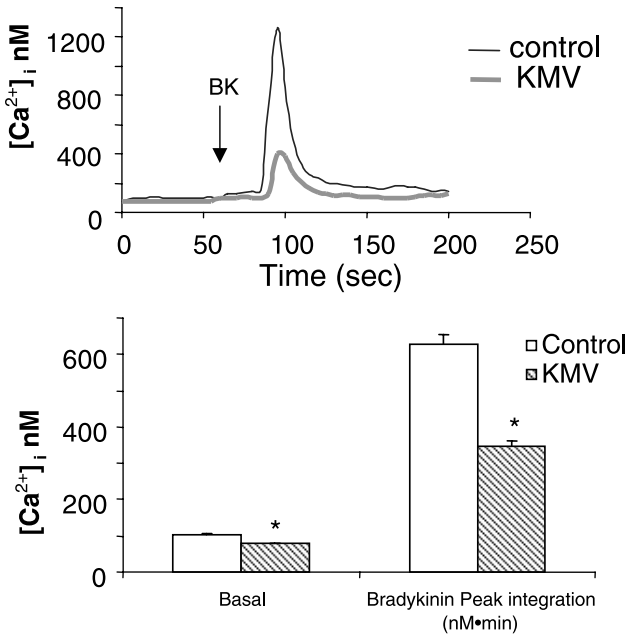

Fig. 4. KMV altered calcium homeostasis in N2a cells. N2a cells were pretreated with KMV for 15 min prior to loading with 2 μM Fura 2AM in the presence of or absence of KMV for 60 min. Controls were treated the same but without addition of KMV. After loading, the medium was washed twice, and exactly 3 min later the resting cytosolic free calcium concentration was monitored for 1 min at 5-s intervals. The top panel shows the temporal response of $[Ca^{2+}]_i$ at resting level and after BK stimulation. The arrow indicates the addition of BK (10 nM). The bottom panel shows the basal level and the integration values of the $[Ca^{2+}]_i$ peak after BK addition. Data are means ± S.E. Each value represents the measurement of 117–157 cells in at least seven dishes measured on 2 separate days. * Denotes KMV differs significantly from the control ($P<0.05$) by Student–Newman–Keul's test.

3.4. Inhibition of KGDHC alters calcium homeostasis

To test whether a partial reduction of KGDHC affects $[Ca^{2+}]_i$ and ER Ca^{2+} stores, N2a cells were treated with 20 mM KMV for 80 min. $[Ca^{2+}]_i$ was monitored for 1 min, and then the cells were stimulated with BK (10 nM). KMV reduced basal $[Ca^{2+}]_i$ by 23% from 101.9 ± 4.1 to 78 ± 2 nM ($P<0.005$; $n=117$ vs. 157), and reduced the BK-induced release of calcium from the ER Ca^{2+} store by 46% from 629 ± 26 to 347 ± 13 nM ($P<0.005$; Fig. 4). Thus, inhibition of KGDHC reduced resting $[Ca^{2+}]_i$ concentration as well as BK-sensitive Ca^{2+} pools.

4. Discussion

The present results demonstrate that a partial inhibition of KGDHC activities in N2a cells by KMV produces no detectable changes in the MMPs, but stimulates release of

cytochrome *c* from mitochondria into cytosol and produces large changes in cellular Ca^{2+} homeostasis.

The present study showed that KMV inhibited KGDHC in situ activity. KMV (20 mM) inhibited KGDHC by 80% (Fig. 1). Although high concentrations of KMV (mM) are required to reduce cellular KGDHC activities, the inhibition is still rather specific. These results agree with previous studies of purified KGDHC [53] and of isolated mitochondria [54]. KMV at 10 and 20 mM effectively inhibits partially purified KGDHC 78% and 99%, respectively [53], as well as in disrupted rat brain mitochondria, but does not inhibit PDHC [53,54].

Impairment of KGDHC was expected to reduce production of NADH by the TCA cycle and thus diminish the MMP, and the subsequent ability of the cell to synthesize ATP. Measurement of KGDHC activity compared to other TCA cycle enzymes suggest it may be rate-limiting in brain slices [54]. This suggestion is supported by the studies of isolated synaptosome showing that inhibition of KGDHC correlates closely with impaired TCA cycle [58]. However, KGDHC is not the rate-limiting enzyme for the TCA cycle in fibroblasts [59]. Surprisingly, inhibition of 80% in situ KGDHC activity does not alter the MMP in N2a cells.

The present study utilizes a new, novel and sensitive technique to quantitatively analyze MMP in single living cells at 37 °C [33]. This method bridges a gap between detailed investigations of individual mitochondria and the approaches that use global fluorescence to study mitochondria. It can detect changes as small as 5%. The lack of change in mean MMP is confirmed by lack of KMV-induced change in the sum of MMP of all pixels of all MLP for each cell. The results suggest that under these experimental conditions, KGDHC activity is not limiting for mitochondrial ability to maintain the MMP. One possible mechanism that can maintain this potential is that the glucose maintains the MMP by reversal of the ATP synthetase. Normally, ATP synthetase uses the MMP to form ATP. However, MMP can be maintained by the level of cytosolic ATP produced by glycolysis when the respiration is blocked [40,60]. Regardless of the mechanism for maintenance the MMP, the results show that inhibition of KGDHC causes profound changes in cellular functions.

The lack of change in MMP indicates that the release of cytochrome *c* by KMV does not depend on a reduction in MMP. The molecular mechanism responsible for the translocation of cytochrome *c* from mitochondria into cytosol is unknown. Although cytochrome *c* release from the mitochondria has often been associated with diminished MMP [61–64], cytochrome *c* release can also occur prior to the loss of MMP or independent of the MMP [30,41,44,65] or as a result of loss of the inner MMP [22,64,66]. A recent study using single-cell analysis shows a transient loss of MMP after cytochrome *c* release that is not apparent in global cell analysis [42]. The present study measures MMP on an individual cell basis, but transient alterations in the MMP will not be apparent. Furthermore, the cytochrome *c* release is assessed for the whole population of cells. Studies in isolated liver mitochondria show that reduction of KGDHC facilitates the mitochondrial permeability transition (MPT) and the effect is independent of the MMP [67]. Whether the MPT is coupled to MMP is controversial [39,40,45]. Regardless of the mechanism, the present studies demonstrate that a large reduction in KGDHC activities induces the release of cytochrome *c* from mitochondria into cytosol without altering the MMP in N2a cells.

One of the goals of these studies was to determine whether or not a reduction in KGDHC can lead to increased ER Ca^{2+} store that accompanies AD-bearing mutations. ER Ca^{2+} stores are linked to a variety of neurodegenerative processes [68–71]. Bombesin-sensitive Ca^{2+} stores are exaggerated in fibroblasts from AD patients as well as in the neurons from PS1-transgenic mice [72]. In addition, transfection of PS-1 in PC12 cells exaggerates Ca^{2+} responses to carbachol or BK that induce Ca^{2+} release from ER [73]. However, the present study shows that inhibition of KGDHC activity reduces basal $[Ca^{2+}]_i$ and the ER Ca^{2+} stores, i.e. the opposite direction of those observed in AD patients or in cells transfected with PS-1. Thus, the results indicate that the reduction in KGDHC does not lead to the calcium changes that accompany AD-causing mutations.

The link between reduced KGDHC, ER Ca^{2+} stores and cytochrome *c* release is unknown, but free radical regulation provides a plausible mechanistic link. Prominent thiol groups in KGDHC subunits support cellular antioxidant capacity through thioredoxin [74]. In thioredoxin-deficient cells, cytochrome *c* is released even though the MMP is maintained [75], i.e. a result similar to the current results. One possible mechanism of cytochrome *c* release is via opening of the mitochondrial permeability transition pore (MPTP). The MPTP can also be regulated by disulfide redox state [76–79]. The dihydrolipoate produced by KGDHC is oxidized by thioredoxin [74], which results in reduced form of thioredoxin. With less dihydrolipoate produced following inhibition of KGDHC, less reduced form of thioredoxin is available. In addition, the reduced form, but not oxidized form, of thioredoxin is able to mobilize both extra- and intracellular Ca^{2+} [80]. Thus, diminished KGDHC will produce less reduced thioredoxin and less Ca^{2+} release from ER. Therefore, the coupling of KGDHC to thioredoxin provides a possible link between the reduction of KGDHC and release of cytochrome *c* and impairment of Ca^{2+} homeostasis.

ER and mitochondria are in close contact. Ca^{2+} cycles communicate between mitochondria and ER Ca^{2+} [81,82]. Mitochondria locally regulate Ca^{2+} flux through IP_3-sensitive channels in the ER [49]. Ca^{2+} released from ER is accumulated locally in microdomains and is taken up by mitochondria prior to generation of change in global $[Ca^{2+}]_i$ [48,83]. The supply of Ca^{2+} from mitochondria regulates Ca^{2+} refilling in ER, and an alteration in the mitochondrial Ca^{2+} interrupts ER Ca^{2+} homeostasis [82]. In addition, three intramitochondrial TCA cycle enzymes (i.e. pyruvate,

KGDHC and NAD^+-dependent isocitrate dehydrogenase) are stimulated by Ca^{2+} [84]. Activation of these enzymes by Ca^{2+} can thus stimulate ATP synthesis [48]. KMV inhibits KGDHC activity 80% which may decrease Ca^{2+} uptake to the mitochondria and into the ER, and this will lead to leakage of calcium from the cell and diminished $[Ca^{2+}]_i$.

In summary, the present results demonstrate that an 80% reduction of KGDHC does not produce detectable changes in the MMP but activates cytochrome *c* release from the mitochondria. In addition, inhibition of KGDHC dramatically reduces the cellular calcium stores that are exaggerated in cells from Alzheimer patients or in cells bearing presenilin mutations. This result suggest that reduction of KGDHC is not the primary cause for the increase Ca^{2+} response in AD. Inhibition of KGDHC with less reduced form of thioredoxin may link the reduction in KGDHC to ER Ca^{2+} homeostasis and cytochrome *c* release.

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